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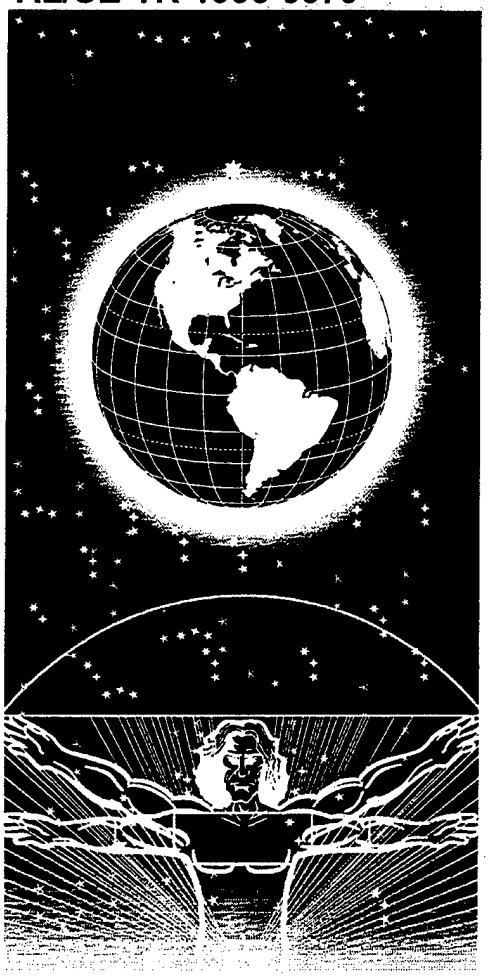

UNITED STATES AIR FORCE ARMSTRONG LABORATORY

TRICHLOROETHYLENE: FREE RADICAL STUDIES IN B6C3F1 MOUSE LIVER SLICES

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The animal use described in this study was conducted in accordance with the principles stated in the "Guide for the Care and Use of Laboratory Animals", National Research Council, 1996, and the Animal Welfare Act of 1966, as amended.

This report has been reviewed by the Office of Public Affairs (PA) and is releasable to the National Technical Information Service (NTIS). At NTIS, it will be available to the general public, including foreign nations.

This technical report has been reviewed and is approved for publication.

FOR THE DIRECTOR

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Electron paramagnetic resonance EPR/spin techniques were used to test the hypothesis exposure of liver to trichloroethylene (TCE) causes increased formation of free radicals. Precision cut liver slices from B6C3F1 mice were incubated for 20 min. with TCE at headspace concentrations ranging from 0-10,000 ppm with and without the spin trap N-tert-butyl-a-phenyl nitrone (PBN). PBN (10 mM) did not interfere with K+ levels, lactate dehydrogenase, aspartamine transaminase or alanine transaminase. Free radicals were measured using Bruker ESP300E spectrometer. A solution of 3-carbamoyl-2,2,5,5,tetra methyl-1-yloxyl free radical was used as a standard to quantitate radicals. All data was normalized by liver wet weight. Total radicals increased linearly with increasing TCE headspace concentration (TR={4.9E-04[TCE]+6.84} X 10E14, r=0.99 P<0.001). EPR/spin trapping results were corroborated by conjugated diene measurements and analysis by gas chromatography.

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PREFACE

This is one of a series of technical reports generated for the Free Radical Studies of Trichloroethylene funded by the Air Force Office of Scientific Research. The research presented here began in February 1994 and was completed in September 1994. The results were partially presented at the International Society of Free Radicals Conference in Sydney, Australia in November 1994 and at the Society of Toxicology Meeting in Baltimore in March 1995. This research was part of the Environmental Initiative project, work unit # 231ZA202 managed by Maj. SR Channel, USAF, BSC. Lt Col Terry Childress served as Contract Technical Monitor for the U.S. Air Force, Toxicology Division.

The animals used in this study were cared for under the *Guide for the Care and Use of Laboratory Animals*, prepared by the committee on Care and Use of Laboratory Animals in the Institute of Laboratory Resources, National Research Council, Department of Health and Human Services, National Institute of Health Publication # 86-23, 1985, and the Animal Welfare Act of 1966, as amended.

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ABBREVIATIONS

ANOVA	Analysis of variance analysis
3-CAR	2,2,5,5,-tetramethyl-1-pyrolidinyl oxy-3-carboxamide
dB	decibel
EPR	electron paramagnetic resonance
G	Gauss
mM	millimole/liter
ms	millisecond
mW	milliwatt
PBN	N-tert-butyl-α phenyl nitrone
TCE	trichloroethylene

INTRODUCTION

Trichloroethylene (TCE) is a widely used industrial solvent and degreasing agent and is an environmental contaminant in the ground water near landfills and in highly industralized areas (Atlas 1995). TCE is lipophilic and can diffuse quickly through hepatic parenchyma cell membranes where it is extensively metabolized. Ingestion of this halogen compound causes liver toxicity in B6C3F1 mice (NCI 1976, Elcombe 1985, Dekant et al 1986) and the mechanism (or mechanisms) responsible for this liver toxicity are currently being explored (Channel 1994). The toxic effects of TCE are important from the standpoint of human health and environmental cleanup requirements. Our aim was to perform as many tests as possible (free radical production, viability tests, TCE metabolism) in the same sample of mouse liver to reduce numbers of animals and experimental variation from one experiment to another. This is possible by preparing slices of liver of equal size and thickness and placing it in incubation media containing the chemical of interest (Smith et al 1987). Electron paramagnetic resonance (EPR)/spin trapping techniques are the only definitive methods to study free radicals (Carmichael et al 1993, Carmichael and Steel-Goodwin 1994). EPR/spin trapping techniques were used to examine free radical formation by liver metabolism of TCE in aqueous media. Spin trapping is a technique in which short-lived free radicals are accumulated by an addition reaction to a spin trap generating a long-lived radical product (Mason 1984). This spin adduct is detected by EPR. Understanding of TCE-induced radicals in aqueous media has

potential application for TCE risk assessment and for applied technology for removal of TCE from soil and ground water (Atlas 1995).

METHODS AND MATERIALS

Liver slice preparation

Precision cut liver slices from B6C3F1 mice were pre-incubated for 2 h in Waymouth's control media prior to incubation in control media or media supplemented with 0.01M N-tert-butyl-α-phenyl nitrone, PBN (Sigma, St. Louis MO.). TCE (Aldrich, Milwaukee, WI) was added to the headspace of the media at concentrations of 0, 2500, 5000, 7500, or 10,000 ppm TCE. After 20 min. the liver slices and incubate were harvested for free radical, conjugated dienes, viability and gas chromatography analysis.

Free radicals

Free radicals were measured using a Bruker ESP300E spectrometer. The magnetic field was set at: 335.5 mT; microwave frequency: 9.4 GHz; microwave power: 20 mW; modulation amplitude: 0.1 mT; time constant: 0.5 s; scan time: 4 min.; scan range 10.0 mT. The samples were quantitated using a standard solution of 3-carbamoyl-2,2,5,5,tetra methyl-1-yloxyl (3-CAR) radical dissolved in bicarbonate solution and lyophilized in the same manner as the liver incubate media.

Conjugated dienes

Conjugated diene concentration was calculated from a standard curve of absorbance of known octadecadienoic acid concentrations (Sigma Chemical Co., St. Louis, MO) read at 235 nm.

Viability

Viability was determined by measuring intracellular potassium content and leakage of the liver enzymes lactate dehydrogenase (LDH), aspartamine transaminase (AST) and alanine transaminase (ALT).

Gas Chromatography

Gas chromatography was used to measure TCE added to the headspace, the partition coefficient of TCE between the headspace and the media, TCE in the media, trichloroethanol and trichloroacetic acid by a modification of the method of Garrett and Lambert 1966).

Statistics

All results were normalized by liver wet weight and analyzed by analysis of variance.

RESULTS

Figures 1-4 describe the formation of radicals after liver slices are exposed to TCE and the subsequent formation of conjugated dienes which suggests TCE induced lipid peroxidation. Tables I & II show the data obtained using established enzymatic and photometry techniques in the laboratory for viability (liver enzymes and K^+) and for TCE and TCE-metabolite quantitation.

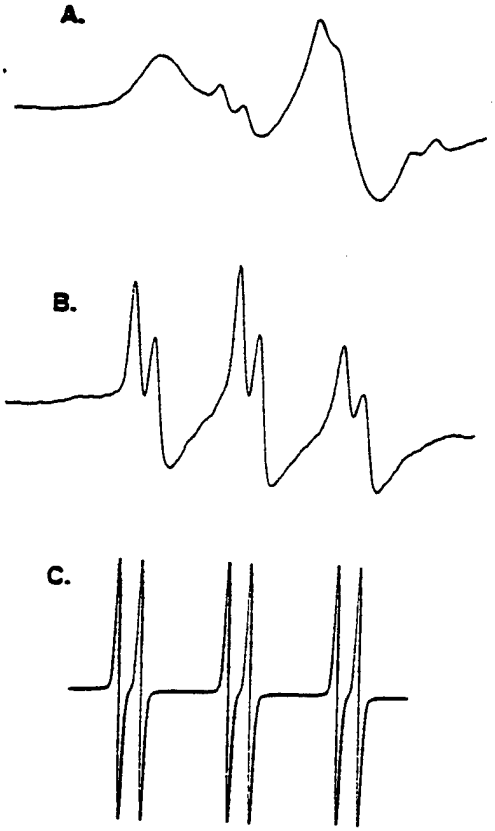

Figure 1. ***EPR spectra. (A) Lyophilized liver incubate after 20 min. exposure to 2500ppm TCE. (B) Spectrum after dissolving lyophilized incubate in water (C) Computer simulated spectrum of B.***

Figure IA is typical of a nitroxide EPR spectrum in solid matrix. This shows the PBN spin adduct EPR spectrum of lyophilized liver incubate after 20 min. exposure to 2500 ppm TCE. The PBN spin adduct EPR spectrum after dissolving the lyophilized liver incubate in water is shown in Figure 1B. Samples generated EPR spectra consisting of a triplet of doublets characteristic of PBN spin adducts. The computer generated EPR spectrum for the EPR spectrum in B is shown in Figure 1C. This spectrum was generated using hyperfine coupling constants a_N=1.61 mT and a_H = 0.325 mT.

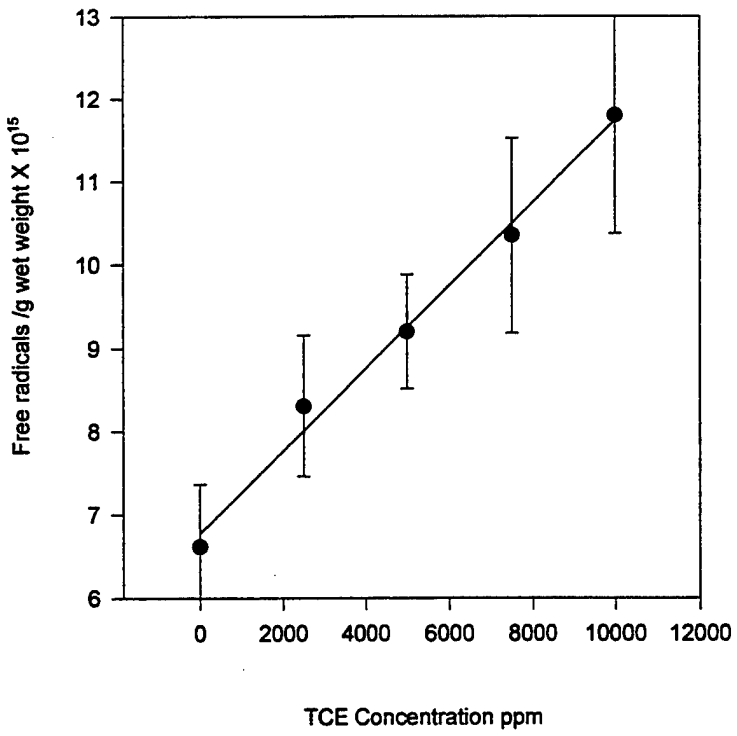

Figure 2 ***Correlation analysis of PBN-spin adducts between TCE concentration and free radicals generated.***

The radicals generated in the incubate of liver samples exposed to 0-10,000 ppm TCE were quantitated using 3-CAR as a standard are shown in Figure 2. Correlation analysis of PBN-spin adducts quantitated using 3-CAR determined a significant link between TCE concentration and free radicals generated ($r = 0.99$ $P < 0.001$).

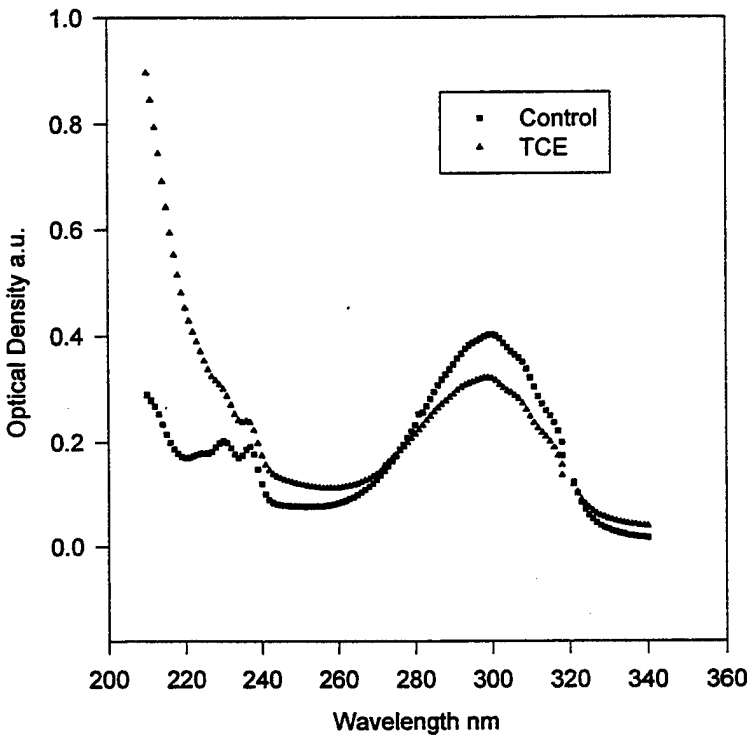

Figure 3 ***Absorbance spectrum of conjugated dienes of liver exposed to 0 or 5000 ppm trichloroethylene.***

Conjugated dienes were measured in the liver slices in these experiments. The typical spectra is shown in Figure 3. The absorbance at 235 nm was used to quantitate the conjugated dienes in the liver slices following exposure to 0-10,000 ppm TCE.

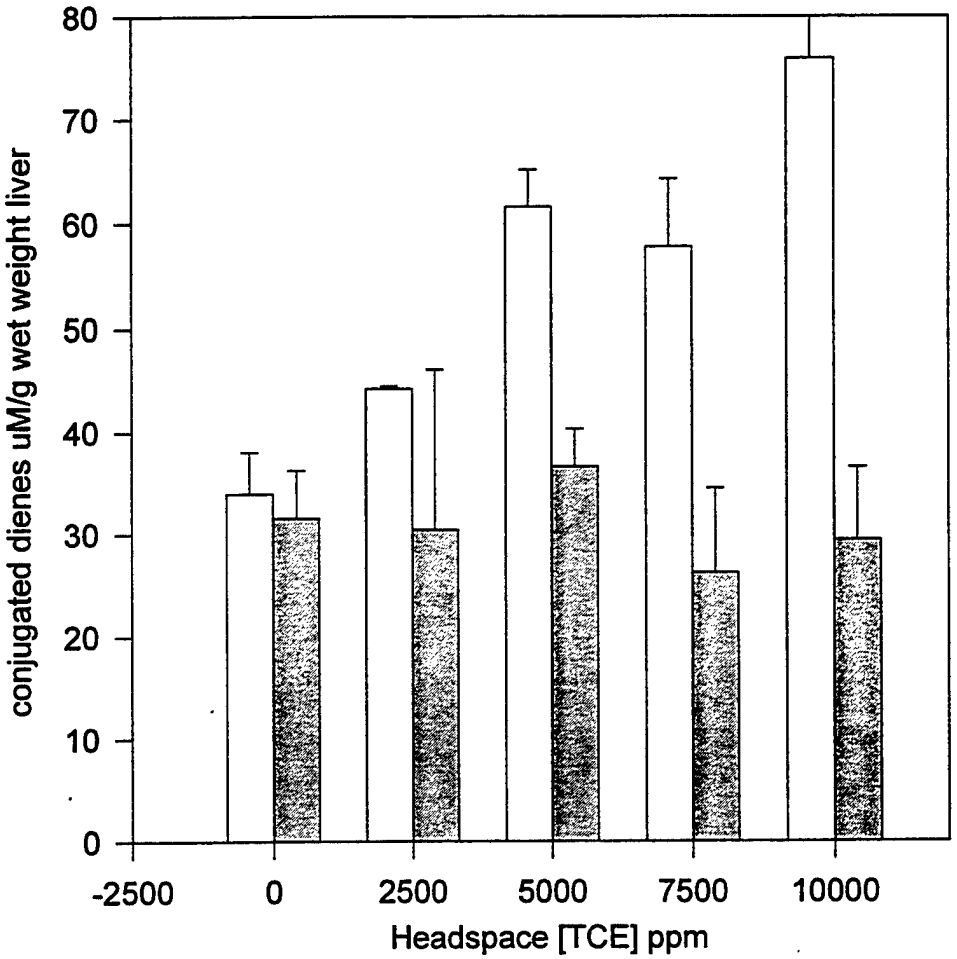

Figure 4 ***Conjugated diene measured from 0-10,000 ppm in liver slices incubated ± 10 mM PBN.***

Generation of free radicals in liver is known to cause lipid peroxidation which can be determined by measurement of conjugated dienes or malondialdehyde (MDA). In these experiments attempts to measure MDA were abandoned because the spin trap

reduced the levels of MDA to levels below detection for the assay. However the levels of conjugated diene measured indicated that as TCE concentration increased, there was a concomitant increase in the concentration of TCE in the control media. The diene concentrations remained the same in the PBN-media suggesting sufficient PBN was present to trap any radicals produced by the liver slices.

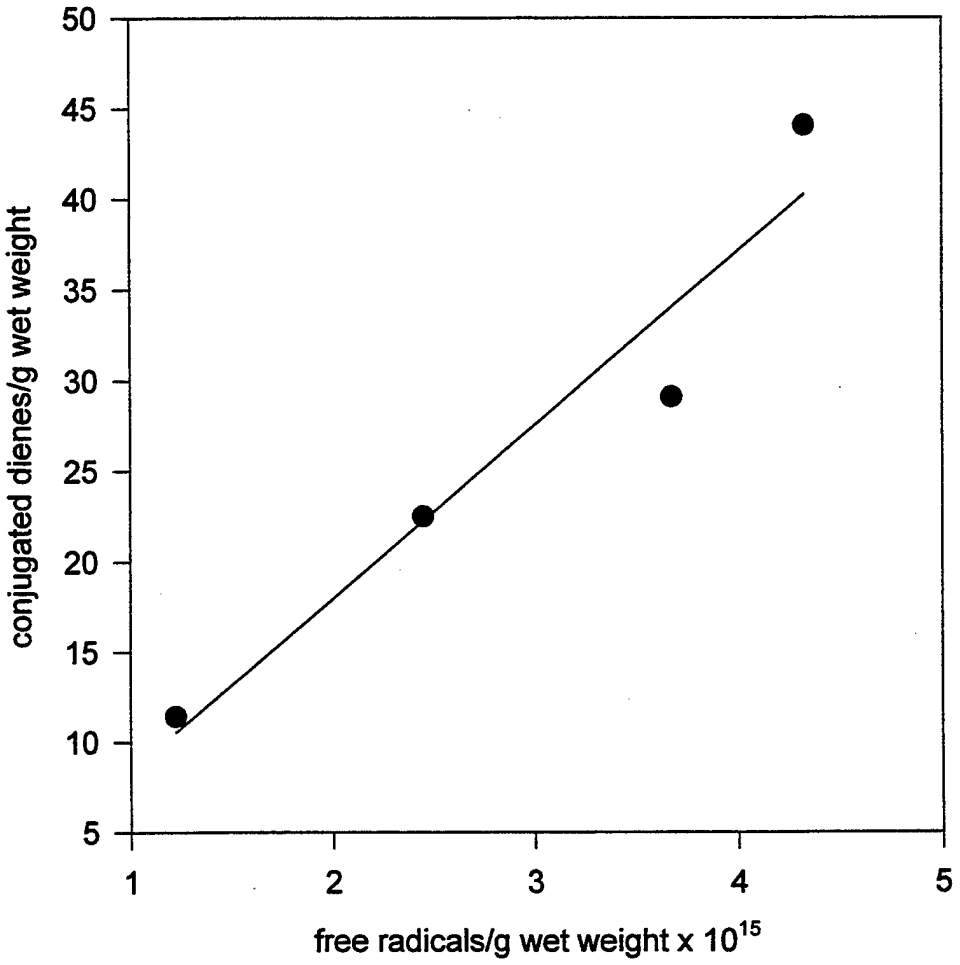

Figure 5 ***Comparison of free radicals trapped with conjugated dienes.***

Figure 5 is the comparison of the data for the free radicals trapped by PBN (Figure 2) with the difference in conjugated dienes ± spin trap (Figure 4). The resulting line was linear (b[0] =-1.1644, b[1] =9.5679, $r^2 = 0.9289$)

This data was dependent on the assumption that the TCE used in the experiments did not cause any adverse changes in the liver during the course of the experiment. The viability of the liver was determined by measurement of liver enzyme leakage and K^+ leakage. Table I is a summary of the measurements of viability.

TCE ppm	Media	Slices	% ALT	% AST	%LDH	K^+
0	-PBN	n = 22	6 ± 2	1 ± 1	9 ± 3	54 ± 16
0	+PBN	n = 12	7 ± 2	1 ± 0.2	10 ± 2	53 ± 7
2500	-PBN	n = 12	6 ± 1	1 ± 0.3	8 ± 1	63 ± 14
2500	+PBN	n = 12	6 ± 3	1 ± 0.5	10 ± 4	59 ± 5
5000	-PBN	n = 12	6 ± 4	2 ± 1	9 ± 6	49 ± 22
5000	+PBN	n = 12	5 ± 2	1 ± 0.3	8 ± 3	60 ± 4
7500	-PBN	n = 12	7 ± 2	2 ± 0.3	10 ± 4	58 ± 18
7500	+PBN	n = 12	6 ± 1	1 ± 0.2	10 ± 1	57 ± 5
10000	-PBN	n = 18	7 ± 2	2 ± 0.6	10 ± 2	59 ± 13
10000	+PBN	n = 12	6 ± 1	2 ± 0.4	10 ± 2	51 ± 2

Table I ***Viability determination of liver slices exposed to 0-10,000 ppm TCE over 20 min. ± PBN.***

Based on enzyme and K^+ leakage tests the liver slices are viable regardless of the TCE concentration and incubation media. Figures 1 & 2 and Table I suggest radicals formed by liver metabolism of TCE in control media induce lipid peroxidation at levels which are not cytotoxic.

TCE ppm	Media	Slices	TCE ppm added	TCE ug/g liver	TCOH ug/g liver	TCA ug/g liver
0	-PBN	n = 8	0	0	0	0
0	+PBN	n = 8	0	0	0	0
2500	-PBN	n = 8	2460	27±3	116±16	49±4
2500	+PBN	n = 8	2339	120±38*	142±14	31±4*
5000	-PBN	n = 8	5057	98±1	130±12	82±4
5000	+PBN	n = 8	5169	274±27*	141±8	32±4*
7500	-PBN	n = 8	7505	177±10	181±13	54±5
7500	+PBN	n = 8	7646	302±39*	177±10	44±2
10000	-PBN	n = 8	9977	171±18	155±15	61±7
10000	+PBN	n = 8	9846	500±3*	163±23	42±2

* P< 0.05

Partition Coefficient $-PBN_{media:air}$ 2.45

Partition Coefficient $+PBN_{media:air}$ 2.24±0.24 (mean±SD)

Table II. ***Metabolism of TCE by liver slices ± 10 mM PBN.***

Table II is the data of TCE metabolism in the control and PBN-supplemented media measured by gas chromatography (GC). Although TCE added to the headspace and the partition coefficients determined in both media were the same, the amount of TCE recovered at the end of 20 min. was greater in the PBN-media than in the control. TCE is believed to be metabolized through free radical pathways and despite being at the limits of detection of the metabolites there was significantly more ($P<0.05$) TCA measured in the control media than the PBN media at headspace concentrations of 2500

and 5000 ppm TCE. In these experiments there was no difference in the TCOH recovered.

DISCUSSION

The objectives of this study were: (1) determine if free radicals and their subsequent biological effect (lipid peroxidation) could be quantitated in B6C3F1 mice after TCE exposure and (2) corroborate data with established laboratory techniques.

The results in Figures 1 and 2 show that free radicals are generated in aqueous media when liver slices are exposed to TCE and that there is a correlation between these radicals and oxidative stress. It is important to stress that radicals were measured in aqueous media. Previous studies of radical effect of TCE have been carried out in non-polar solvents (Gronthier et al 1989 and Stevens 1994). The majority of biological reactions occur in the aqueous phase of the cell. (von Sonntag 1991). It is therefore important to study radical induced reactions in this phase. In this study 3-CAR was used to quantitate the radicals trapped by PBN. However the radicals trapped by PBN are at most only 20 % of the total radicals in the liver incubate (Steel-Goodwin and Carmichael 1995). In this project it is concluded that liver metabolism of TCE generates PBN spin adducts in aqueous media with hyperfine coupling constants of a_N = 1.61 mT and a_H = 0.325 mT.

Lipid peroxidation in biological systems involves complicated reactions in which conjugated dienes are only part of the cascade (Rice-Evans et al 1991). Attempts to established a relationship between TCE addition and malondialdehyde, the main defined lipid peroxidation reaction product measurable in biological systems were not pursued but initial experiments showed no detectable radicals in the PBN-media (results not shown). Radical-induced lipid peroxidation (oxidation of polyunsaturated fatty acids) has

mainly been studied in organic solvents but the difference of radicals quantitated with and without TCE in the aqueous media correlates with the oxidative stress measured from the dienes with and without the spin trap.

The results in Table I and II suggest TCE-generated radicals are selective in their reactions. For example, the TCE-induced radicals were not reactive enough to alter the viability of liver cell membranes at the TCE head space concentrations 2500-10,000 ppm. However the TCE concentration was sufficient to increase in conjugated dienes.

Halogenated hydrocarbons: carbon tetrachloride, trichloroethylene and bromobenzene do induce toxic effects on cells (Steup et al 1991, Ashby et al 1994) but lipid peroxidation (Comporti, 1987., Recknagel et al 1987., Fraga et al 1989) may only be a secondary event, because of cell damage induced by the majority of toxins (Halliwell et al 1992., Gutteridge 1995).

The results shown in Table II suggest free radicals are involved in the liver metabolism of TCE by B6C3F1 mice. The disappearance of TCE at concentrations of 0-10,000 ppm was inhibited when the liver slices were incubated in the spin trap PBN. The results further suggest that the slices exposed to spin trap produced less TCA . Free radical pathways of TCA and DCA production are currently being studied by Stevens, 1994 and there study will not be duplicated. In the present study, the spin trap did not alter the production of TCOH. B6C3F1 mice are a strain which is sensitive to adverse TCE effects, however, free radical studies in human slices could provide important information on potential adverse effects free radicals may cause following TCE exposure. Humans are normally exposed to TCE in low doses in drinking water. Drinking water

resources are finite and TCE-contaminated water, tastes bad. As TCE-induced radicals are generated in aqueous media, radical forming agents could be used to rehabilitate former industrial sites and remove TCE from ground water. A number of techniques are currently being used to reduce TCE in drinking water (Atlas 1995) and these methods may be successful because of their effects on free radicals. Information on free radical effects of TCE could prove useful for studies of potential risks to humans. The risk of potential adverse health effects to humans following exposure to TCE contaminated water supplies is currently based on PD models (Fisher et al 1991 and Fisher and Allen 1993). These models are based on measurements of parent compound and metabolites which have been determined by GC techniques so the GC data, Table II, can in theory be compared to these PD models. In addition, the new parameters: free radicals and conjugated dienes can be added to the model.

Finally, as this study determined that the liver slices were viable, Table I, the results, Figures1-5, strongly suggest TCE breaks down in liver slices producing free radicals which induce lipid peroxidation. Should this occur *in vivo* in the mouse liver, the resulting lipid peroxidation cascade could lead to impairment of cell junctions or cell death or malignant transformations.

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